PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12Q 1/68, G01N 33/543, 21/77

A1

(11) International Publication Number: WO 93/06241

(43) International Publication Date: 1 April 1993 (01.04.93)

(21) International Application Number: PCT/GB92/01698

(22) International Filing Date: 16 September 1992 (16.09.92)

(30) Priority data:
9119735.0 16 September 1991 (16.09.91) GB

(71) Applicant *(for all designated States except US)*: THE SECRETARY OF STATE FOR DEFENCE IN HER BRITANNIC MAJESTY'S GOVERNMENT OF THE UNITED KINGDOM OF GREAT BRITAIN AND NORTHERN IRELAND .[GB/GB]; Whitehall, London SW1A 2HB (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)* : SQUIRRELL, David, James [GB/GB]; CBDE, Porton Down, Salisbury, Wiltshire SP4 0JQ (GB).

(74) Agent: P. B. LOCKWOOD; MOD(PE), DIPR, Empress State Building, Room 2121; Lillie Road, London SW6 1TR (GB).

(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, RU, SD, SE, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

Published
With international search report.

(54) Title: GENE PROBE BIOSENSOR METHOD

(57) Abstract

The present invention relates to a method for the detection, identification and/or quantification of plant or animal tissues, microorganisms or cell free RNA or DNA and to detector apparatus adapted for performing said method. The method particularly uses Total Internal Reflection Fluorescence (TIRF) to measure hybridization of analyte RNA or DNA with RNA or DNA that is associated with an evanescent wave detector waveguide.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	MN	Mongolia
AU	Australia	FR	France	MR	Mauritania
BB	Barbados	GA	Gabon	MW	Malawi
BE	Belgium	GB	United Kingdom	NL	Netherlands
BF	Burkina Faso	GN	Guinea	NO	Norway
BG	Bulgaria	GR	Greece	NZ	New Zealand
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	PT	Portugal
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	RU	Russian Federation
CG	Congo	KP	Democratic People's Republic of Korea	SD	Sudan
CH	Switzerland			SE	Sweden
CI	Côte d'Ivoire	KR	Republic of Korea	SK	Slovak Republic
CM	Cameroon	LI	Liechtenstein	SN	Senegal
CS	Czechoslovakia	LK	Sri Lanka	SU	Soviet Union
CZ	Czech Republic	LU	Luxembourg	TD	Chad
DE	Germany	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America

GENE PROBE BIOSENSOR METHOD.

The present invention relates a method for the detection, identification and/or quantification of plant or animal tissues, microorganisms or cell free RNA or DNA and to reagents and detector apparatus adapted for performing said method. The method particularly uses Total Internal Reflection Fluoresence (TIRF) to measure hybridization of analyte RNA or DNA with RNA or DNA that is associated with an evanescent wave detector waveguide.

Gene probe assays, using nucleic acid hybridization, are an alternative to immunoassays in the detection and identification of biological materials. The specificity of gene probes for their targets can be controlled much more easily than is possible using protein-based binding phenomena and, when coupled with the polymerase chain reaction to pre-amplify the target material, extreme sensitivity can be obtained. Current techniques using gene probes are slow, taking from hours to days to produce a result. Biosensors offer an alternative route to fast gene probe assays, but the only reports so far on gene probe biosensor assays are those using surface plasmon resonance (Evans & Charles (1990); Abstracts of 1st World Congress on DNA probes and immunoassay; Pollard-Knight et al (1990) Ann. Biol. Clin, 48 642-646).

Evanescent wave biosensors, which use the phenomenon of TIRF for detection (Sutherland & Dahne, (1987) J. Immunol.Meth., 74, 253-265), have previously been used with proteins as the biological recognition element. Antibodies have been used to detect the binding of fluorescent-labelled antigen (Eldefrawi et al (1991) Biosensors & Bioelectronics, 6, 507-516) using acetylcholine receptors to study the binding of acetylcholine and cholinesterase inhibitors. Other groups (Poglitsch & Thompson (1990) Biochemistry, 29, 248-254) have measured the binding of antibody to F_c epitopes.

The present invention provides a method for carrying out gene probe assays with an evanescent wave biosensor and provides a TIRF waveguide which is adapted for carrying out said method when incorporated within an evanescent wave biosensor device.

Evanescent wave detectors exploit the TIRF phenomenon to provide a sensitive method for detecting reactions at the surface of waveguides. The waveguide may take various forms but typically will be a prism, slab or fibre. The reaction to be used to measure the target molecule may be monitored, for example, through measuring the fluorescence changes on binding or desorption of fluorescent species or by the generation of fluorescent species by enzymic or chemical means. Several patents have been published that cover the use of evanescent wave detectors with immunoassay systems as the biological sensing element (eg. US 4582809) but the inherent limitations in the immuno-reagents have not allowed the full capabilities of the sensor to be exploited.

The present invention provides a method for the detection, identification and/or quantification of a material selected fom plant or animal tissue, microorganisms or cell free RNA or DNA comprising:

(i) providing an oligonucleotide, complementary to all or part of an oligonucleotide sequence characteristic of the RNA or DNA of the material, immobilised on the surface of an evanescent wave detector apparatus waveguide;

(ii) exposing the immobilised oligonucleotide of step (i) to the sample, or DNA or RNA material derived therefrom, under conditions whereby DNA or RNA having the characteristic oligonucleotide sequence will hybridize with it;

(iii) associating the immobilised oligonucleotide of step (i) or the nucleic acid material derived from the sample with a fluorescently detectable agent, before, during or on completion of any hybridization with DNA or RNA as provided in step (ii), such that the fluorescently detectable agent becomes bound to the hybridized product but not to unhybridized immobilized oligonucleotide;

(iv) measuring the fluorescently detectable agent bound by step (iii) using the evanescent wave detector apparatus and relating the amount of that to the presence, identity and/or amount of the material.

Sandwich assays Not found for immobilised

In one preferred form of the present invention the fluorescently detectable agent is a fluorescently detectable intercalating dye capable of being incorporated into the duplex of the hybridization product. In this form of the method the dye is one that does not bind to the unhybridized immobilised oligonucleotides. Such method of determining the presence of hybridization product is ideally used with immobilised sequences of sufficient length in themselves to provide specific hybridization under the hybridization conditions used. The sample so analysed may be unaltered in DNA/RNA content, or may have had this enriched by specific sequence amplification, eg. polymerase chain reaction amplification (PCR) or ligase chain reaction (LCR).

In a second preferred form the fluorescently detectable agent is a fluorescently detectable oligonucleotide which is capable of hybridizing with the hybridized characteristic sequence or which is capable of acting as a specific sequence amplification primer, eg. such as a polymerase chain reaction primer, to yield fluorescently detectable amplification product, which is detectable using evanescence when hybridized to the waveguide immobilised oligonucleotide.

2ND embodiment ↳ labelled target

In a preferred embodiment of the second form, using detectable oligonucleotide, the present invention provides a method for the detection, identification and/or quantification of a material selected from plant or animal tissue, microorganisms or cell free RNA or DNA comprising:

(i) providing an oligonucleotide, complementary to part of an oligonucleotide sequence characteristic of the RNA or DNA of the material to be detected, immobilised on the surface of an evanescent detector apparatus waveguide;

(ii) providing a fluorescently detectable oligonucleotide complementary to all or part of the remainder of the characteristic sequence;

(iii) exposing the immobilised oligonucleotide from (i) to a solution comprising material to be investigated, or DNA or RNA material derived therefrom, under conditions whereby characteristic oligonucleotide

sequence will hybridize with it;

(iv) replacing the solution of material from (iii) with a solution comprising the fluorescently detectable complementary oligonucleotide from (ii) and

(v) measuring the amount of bound fluorescently detectable oligonucleotide using the evanescent wave detector apparatus and relating that to the presence, identity and/or amount of target material in the sample.

In a further preferred embodiment the present invention provides a method for the detection, identification and/or quantification of, plant or animal tissues, microorganisms or cell free RNA and DNA comprising:

(i) providing an oligonucleotide, complementary to part of an oligonucleotide sequence characteristic of the RNA or DNA of the material to be detected, immobilised on the surface of an evanescent wave detector apparatus waveguide;

(ii) providing a fluorescently detectable oligonucleotide identical to all or part of the remainder of the characteristic sequence;

(iii) performing a specific sequence amplification reaction on a sample of the material under investigation using the fluorescently detectable oligonucleotide of step (ii) as one of the primers, to thereby amplify any of the characteristic sequence present while incorporating fluorescently detectable agent therein;

(iv) exposing the waveguide immobilised complementary oligonucleotide of step (i) to the reaction mixture from step (iii) under conditions whereby hybridization of the amplification product with said immobilised oligonucleotide will occur;

(v) measuring any fluorescently detectable oligonucleotide hybridized with the immobilised oligonucleotide using the evanescent wave detector and relating that to the amount of amplified material

initially present in the sample.

Preferably and conveniently the specific amplification reaction of step (iii) is a polymerase chain reaction, but other such reactions such as the ligase chain reaction may usefully be employed, as will be clear to those skilled in the art.

In all of the embodiments of the invention the waveguide is preferably situated within a temperature controllable environment, eg. within a temperature regulated chamber, such that the stringency of the hybridization of the complementary sequences to the target RNA or DNA may be controlled, or such that the polymerase chain reaction denaturation, hybridization and extension temperature cycles might be carried out.

The fluorescently detectable complementary oligonucleotides may be provided in a number of forms, eg. as an oligonucleotide tagged either with a fluorescent label, with a moiety to which a fluorescent species can be bound or with an enzyme or catalyst capable of generating fluorescent species. Thus step measuring steps may require additional steps prior to the basic fluoresence detection step, eg: exposure of the moiety to which a fluorescent species may be bound to such species or by exposure to substrate for the enzyme or catalyst reaction.

The complemetary sequence of step (i) may be of sufficient length to specifically bind the target RNA or DNA whilst leaving enough unbound target RNA or DNA sequence to bind the detectable oligonucleotide of step (iii). Typically the immobilised sequence will comprise about half of the length of the target sequence, but increasing the length of target sequence will allow lesser proportions to be used.

In a preferred embodiment employing the detectable oligonucleotide, the detectable sequence of will be complementary to all or almost all of the remainder of the target sequence but this may similarly be a reduced portion as target characteristic sequence length increases.

Advantageously the immobilised oligonucleotide is complementary to a sequence toward or at one end of the target sequence while the

fluorescently detectable sequence is complementary to a sequence toward of at the other end. Thus restriction endonucleases may be conveniently employed prior to the hybridization for the purpose of cleaving the RNA or DNA of the sample material in order to allow easier access to these ends of the characteristic target sequence. Similarly in the second preferred embodiment the detectable sequence need only be long enough to be useful as a PCR primer of the necessary specificity but may be longer. It will be appreciated that both PCR primers may be fluorescently detectable thus increasing the potential fluorescence provided at the waveguide surface and thus the efficacy corresponding evanescent effect. In all cases preferred reagent baselengths are 5-30, more preferably 10-25, eg. 15-20 bases.

It will be realised by the man skilled in the art that the complementary sequences need not be complemetary at every base pair where they hybridize. Thus a man skilled in the art will readily determine the stringency requirements for statistically acceptable use of mismatched oligomers eg. 90% complementary sequences and will be able to obtain usable results by adjusting the hybridization temperature accordingly where such mismatched sequences are used.

The requirements for precise complementary sequence usage will vary with the uniqueness of the sequence for which the user is assaying. Thus a sequence that shares a high percentage of bases with other potentially present sequences will require use of a more completely complementary sequence than a sequence that is relatively distinct. Thus where the sequence being assayed for is not fully elucidated or is variable, the skilled man may be able to determine statistically acceptable oligomers for steps (i) and (iii).

A second aspect of the present invention provides an assay kit for use in the method of the invention comprising:

(1) an oligonucleotide complementary to all or part of an oligonucleotide sequence characteristic of the RNA or DNA of the material to be detected and (2) a fluorescently detectable oligonucleotide complementary or identical to part or all of the remainder of the characteristic sequence.

The options for the components (1) and (2) are those which are set out above and in the claims, these components being optionally supplemented in the kit by those reagents necessary for immobilisation of the oligomer (1) on the waveguide; examples of suitable reagents being those referred to in the discussion of methods below.

A third aspect of the invention provides a waveguide suitable for use in an evanescent wave biosensor characterised in that it comprises, immobilised upon its surface, an oligonucleotide which is complementary to all or part of an oligonucleotide sequence which is characteristic of the RNA or DNA sequence of the material which is to be detected. Furthermore kits comprising such waveguides are provided for use in the method of the invention, as set out in the claims.

In a fourth aspect of the present invention is provided an evanescent wave detector biosensor characterised in that it comprises a waveguide which has, immobilised upon its surface, an oligonucleotide which is complementary to all or part of an oligonucleotide sequence which is characteristic of the RNA or DNA sequence of the material which is to be detected. Such waveguide may eg. be a prism, slab or fibre.

In all of the aspects of the invention, it will be realised that more than one characteristic sequence may be targeted simultaneously by employing more than one type of immobilised oligonucleotide and associated fluorescently detectable binding agent in the method and apparatus used to carry it out.

Advantages of the present method over analogous immunoassay systems include:

(a) denser packing of the immobilised component on the waveguide surface allowing greater sensitivity and dynamic range and making the construction of multispecificity surfaces easier;

(b) the binding between the analyte and the immobilised and derivatised oligonucleotides can be regulated by adjusting the hybridization conditions and the lengths of the oligonucleotides, thus permitting the specificity and sensitivity of a given test to be finely adjusted to suit requirements;

(c) immunoassays are limited by availability of reagents, stability of reagents, antigenic variability, masking of antigenic sites, and the dissociation constants of the antibody/antigen complexes. Gene probe assays are pontentially superior in all these respects;

(d) fluorescent immunoassays suffer from interference from environmental contaminants and non-specific binding while the higher temperatures required for gene probe assays (ie. above 60°C rather than 37°C) will reduce unwanted adsorption of contaminants on the detector surface;

(e) as a result of the lower dissociation constants obtainable with nucleic acid complexes compared with immune complexes the full sensitivity of evanescent wave detector technology can be realised.

The samples to be investigated may be as found in the environment (eg. for free RNA or DNA oligonucleotides) or may be pretreated such as by cell lysis techniques, DNA/RNA concentration or amplification (eg. as by polymerase chain reaction using Taq polymerase) or by restriction enzyme mediated degredation (eg. to provide more manageable sized oligonucleotides).

The potential of the method, kits and apparatus of the present invention will now be illustrated by way of example only with reference to the following Figures and Experiments; further embodiments will occur to the skilled man in the light of these.

Figures

Figure 1: shows the principal elements of the evanescent wave detector used in the Examples.

Figure 2: shows the voltage/time relationship obtained on hybridization of a fluorescein-labelled 20-mer with a complementary sequence covalently coupled to an optic fibre waveguide.

Figure 3: shows concentration/response curves showing specific hybridization of complementary and control oligonucleotides;

signal(mV/min) plotted against nM concentration oligomer.

Figure 4: shows the effect of flow rate of sample solution throught the temperature controlled chamber containing the waveguide on the hybridization signal; signal (mV/min) is plotted against flow rate (ml/min).

Figure 5: shows the influence of temperature on the rate of hybridization; signal (mV/min) is plotted against temperature °C for a 50ng/ml sample of a fluorescein labelled 20-mer.

Figure 6: shows a pH profile of oligonucleotide hybridization on the optic fibre waveguide; signal (mV/min) plotted against pH.

Figure 7: shows binding from 8nM solutions of oligomers complementary to the proximal or distal ends of a covalently coupled 204 base oligonucleotide.

Figure 8: shows a diagrammatic scheme of the molecular basis of the two of the embodiments of the method of the present invention.

METHODS.

Since oligonucleotides are long-chain molecules with lengths that can be comparable to the depth of the evanescent zone, studies were carried out which hybridized short, fluorescein-labelled oligonucleotides to either end of a 204-base oligomer, a fragment amplified from protective antigen gene of Bacillus anthracis, tethered at one end to the waveguide surface to examine the influence of probe length on detection.

Materials:

Glutaraldehyde, 3-aminopropyl triethoxysilane (APTS), Tween 20, Ficoll, formamide, polyvinyl pyrrolidone, bovine serum albumin fraction V (BSA), horse radish peroxidase labelled streptavidin (HRP-SA), 2,2,-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)

(ABTS), sodium chloride and trisodium citrate were obtained from the Sigma Chemical Company, Poole, UK. Sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate, sodium dodecyl sulphonate (SDS), hydrogen peroxide, hydrochloric acid, and acetone were AR grade from BDH, Poole, UK. Ammonium hydroxide was obtained from Aldrich, Gillingham, Dorset, UK and ethanol from Hayman Ltd., Witham, Essex, UK. Streptavidin immobilised on agarose beads was obtained from Pierce and Warriner, Chester, UK. Synthetic DNA oligonucleotides with C6-linked fluorescein-, amino- or biotin-modified ends were purchased from British Bio-Technology Ltd, Abingdon, UK are listed in Table 1.

Table 1 Oligonucleotides Used (5' to 3')

ID No.	Sequence
4946	CACGTTGTGGACTGTTTGGA-amino
4947	TCCAAACAGTCCACAACGTG-fluorescein
4949	CAACACACCTTAACAC-amino
4950	GTGTTAAGGTGTGTTG-fluorescein
5453	Amino-CTTTAATTGTCGCGAGTGTT
5805	Fluorescein-AACACTCGCGACAATTAAAG
6097	Biotin-AATTCAAGTACGGTCGCAAT
5452	Fluorescein-AATTCAAGTACGGTCGCAAT

The fibre optic evanescent wave device, (prototype No.19), and 1mm x 65mm quartz optic fibres were purchased from ORD, North Salem, New Hampshire, USA. See Figure 1. A six watt tungsten filament source (1) provides light which is passed through three collimating lenses (2) interspaced by a 485mm blue filter (3) and a 0.8mm pinhole mask (4) to impinge upon a 50:50 beam splitting device (5) which intermittently directs light through a focussing lens (6) and into the oligonucleotide bearing waveguide (7). Fluorecsent light derived from any bound, eg. hybridized or intercalated, fluorescent material responding to the incoming light is redirected out of the waveguide (7) via the lens (6) and is allowed to pass through the beam

splitting device toward a 530 nm green filter (8). from here light passes through a shutter assembly (9) and a focussing lens (10) whereafter it impinges upon a detector device (11) connected to a measuring device such as a voltmeter (not shown).

The output voltage from the voltmeter is conveniently connected to a Pharmacia model 482 chart recorder. Optic fibres were held in a jacketed flow cell constructed from glass capillary tubing with stainless steel end caps and silicone gaskets. Temperature was controlled by circulating water from a water bath through the glass jacket. The temperature was monitored using a thermocouple. Optic fibres were cleaned and then covalent coupling with glutaraldehyde was carried out using a method based on that described by Tijssen,(1985), Practice and theory of enzyme immunoassays: page 322 to 323 of Vol 15 of Laboratory Techniques in Biochemistry and Molecular Biology. Ed: Burdon and van Knippenburg: Pub. Elsevier.

Fibres were silanised in a 2% solution of APTS in acetone for 24 hrs at room temperature, washed with acetone, dried at 50°C, immersed in 1% glutaraldehyde in water for 1 hour, washed in PBS and finally left overnight at 4°C in a 10ug/ml solution of oligonucleotide with an aminoterminal. To perform an assay an optic fibre with immobilised oligonucleotide was rinsed in deionised water, blotted dry and inserted into the flow cell. When optimised, the following conditions, adapted from Anderson & Young,(1985) Nucleic acid hybridization - a practical approach Ed. Hames and Higgins; IRL Press, Oxford, pp 73-111, were used. The temperature of the water jacket was adjusted to 65°C.

Prehybridisation solution, (250ml 20xSSC, plus 50ml 100x Denhardt's solution, 50ml 0.1M phosphate buffer and 1ml Tween 20 added to 600ml sterile deionised water, final pH 6.8) was pumped using a peristaltic pump over the fibre in the 25µl flow cell at 0.5ml/min until a steady baseline was obtained. The fluorescein end-labelled target oligonucleotide, in prehybridization solution, was then introduced at the same flow rate and hybridization was followed as the increase in output on thc chart recorder. After a few minutes prehybridization solution was switched back into the flow cell and the temperature raised to 80°C. After 10 to 15 minutes all the bound oligonucleotide

had desorbed and the temperature was lowered to 65°C ready for another run.

Initially only short (16- to 20-mer) oligonucleotides were attached to the fibres. To investigate the use of longer probes an amino-ended 204-mer was synthesised using PCR on the target sequence with one primer, a 20-mer, end-labelled with an amino-group - oligo.5453 - and the other primer, also a 20-mer, end-labelled with biotin oligo.1222. Unincorporated amino-labelled primer was removed using streptavidin immobilised on agarose beads to pull out double-stranded PCR product from unincorporated amino-ended primer. The amino-labelled 204-base strand was released from the complementary strand and the beads by heating at 95°C for 10 minutes followed by quick chilling in a water/ice mixture and then removing the beads by centrifugation. The amount of the 204-mer produced was sufficient for a 0.85µg/ml solution to be used for coupling to fibres.

Assay of oligomers:

In initial 20-mer experiments high levels of target and control oligonucleotides were used. A 200nM (1µg/ml) solution of fluorescein -labelled oligonucleotide control (oligo.4950) was introduced at 1ml/min and 60°C into the flow cell containing a fibre with .oligo.4946 immobilised. There was an immediate shift in baseline of 80mV whereafter the output remained constant until prehybridisation solution was reintroduced when the output returned to its previous level. In contrast, when oligo.4947 which was complementary to oligo .4946 on the fibre was introduced at 160nM, (also 1µg/ml), a continuously rising signal was obtained with an initial slope of about 350mV/min. A plateau was reached after 14 minutes at an output 1,140mV above the baseline level. The output fell again when prehybridization solution at 90°C was passed through the flow cell. To confirm that binding was specific, the control oligo.4950 was shown to bind to an optic fibre with its complement, oligo.4949, covalently coupled to the surface.

The time course of hybridization of 8nM fluorescein-labelled oligo.4947 to immobilised oligo.4946 is shown in Figure 2 and a concentration/response curve for fluorescein-labelled oligo.4950

hybridizing to immobilised oligo.4949 with oligo.4947 as a control is given in Figure 3. The slopes of the curves were measured 1 minute after sample entered the flow cell.

Results from the optimisation of conditions are shown in Figures 4-6. The effect of flow rate was evaluated witn 50ng/ml solutions of oligo.4947. The results are shown in Figure 4. The dependence of the rate of hybridization on temperature and pH were evaluated and the results are shown in Figures 5 & 6,respectively. Maximum rates of binding were observed at 65°C and pH6.8.

The binding curves of 50nM solutions of fluorescein-labelled oligonucleotides complementary to either the proximal or distal ends of a 204-base oligonucleotide covalently coupled at one end to the waveguide surface are shown in Figure 7. There was little difference between responses with both showing a rapid rise to a plateau level of 48mV (proximal) or 56mV (distal) after about three minutes followed by a slight fall in output when prehybridisation solution was reintroduced into the flow cell. Concentration/response curves using initial slopes for hybridization of the two oligonucleotides showed little difference between them, (results not shown). Oligo.4947 at 500nM as a control did not bind to the 204-mer.

Detection was in the nanomolar range with a linear relationship between the slope of the response and concentration : similar to results obtained using immunoassays with this biosensor.

For assays that require high sensitivity, the following equipment improvements and enhancement techniques are conveniently made:

Labelled oligomers are made using oligonucleotides labelled with multiple fluorophores or fluorescent beads, or fluorescent intercalating dyes are used to detect hybridization;

The biosensor assay may is combined with a DNA amplification technique such as PCR which could be performed on the sample, prior to the hybridization on the waveguide, preferably using fluorophore-labelled primers (as in one of the preferred embodiments of the method of this invention above) to improve detection of product. It would also be

possible directly to monitor PCR reactions using the biosensor. Th 60 seconds or so taken for the biosensor assays is orders of magnitude faster than conventional gene probe assays and still much faster than, rapid, tests that take about 1 hour to perform, (Engleberg.(1991) ASM News 57 (4) 183-186).

The easy recycling of the detector surface through removal of bound nucleic acids by heating obviates one of the problems encountered with immunoassays on this system and renders it, with the 60 second response period, useful in military or other critically hazardous environments requiring rapid identification. Several optic fibres were found to be usable over periods of several days with no loss in response.

Both 5' and 3' B,-labelled oligonucleotides were used with this factor had no apparent effect on the results. Oligonucleotides were also bound to the proximal and distal ends of a 204-base oligonucleotide. The length of this could have been from 70nm, (using the value of 3.4nm for the pitch of double-stranded DNA which has 10 bases per helical), to 200nm, (using the inter-bond distances of the -O-P-O-C-C-C- repeats along the backbone of the molecule). The depth of the evanescent zone defined as the distance taken for the light intensity to fall to 1/e of its initial value would have been around 100nm, comparable to the length of the 204-mer. Since similar signals were obtained for the hybridisation of distal and proximal fluorescein-labelled oligonucleotides, (slightly higher signals were obtained for the distal oligonucleotide), the orientation of the long oligonucleotide strands was presumably not perpendicular to the wave guide surface.

Only a limited amount of the 204-mer, which was generated by PCR from a larger target sequence, was available for coupling to the optic fibres and this presumably led to the concentration on the waveguide surface being relatively low. This would explain the rapid plateauing of the responses seen in Figure 7 compared to the continuing rise in signal over an equivalent timescale seen in Figure 2, (where the immobilised gene probe was at a higher concentration, being covalently coupled from a solution >100x more concentrated. If plateau height rather than rate of change of output were to be chosen as the measure

non-fluorophor bearing end of (F) is as shown in Figure 8g.

The intercalating dye utilising embodiments of the method of the invention whilst not specifically exemplified herein, are carried out as illustrated best by Figures 8a and 8b wherein these steps are carried out in the presence or with later addition of the dye. Suitable dyes and conditions for their interaction are disclosed in the prior art, eg. Latt and Wohlleb; Chromosoma (Berl.) 52, 297-316 (1975). Jorgenson et al, Chrmosoma (Berl.) 68, 287-302 (1978) and Jennings and Ridler; Biophysics of Structure and Mechanism, 10, 71-79, (1983).

CLAIMS.

1. A method for the detection, identification and/or quantification of a material selected from plant or animal tissue, microorganisms or cell free RNA or DNA comprising:

(i) providing an oligonucleotide, complementary to all or part of an oligonucleotide sequence characteristic of the RNA or DNA of the material, immobilised on the surface of an evanescent wave detector apparatus waveguide;

(ii) exposing the immobilised oligonucleotide of step (i) to the sample, or DNA or RNA material derived therefrom, under conditions whereby DNA or RNA having the characteristic oligonucleotide sequence will hybridize with it;

(iii) associating the immobilised oligonucleotide of step (i) or the nucleic acid material derived from the sample with a fluorescently detectable agent, before, during or on completion of any hybridization with DNA or RNA as provided in step (ii), such that the fluorescently detectable agent becomes bound to the hybridized product but not to unhybridized immobilized oligonucleotide;

(iv) measuring the fluorescently detectable agent bound by step (iii) using the evanescent wave detector apparatus and relating the amount of that to the presence, identity and/or amount of the material.

2. A method as claimed in claim 1 wherein the fluorescently detectable agent is a fluorescently detectable intercalating dye capable of being incorporated into the duplex of the hybridization product; that being a dye that does not bind to the unhybridized immobilised oligonucleotides.

3. A method as claimed in claim 1 or claim 2 wherein the characteristic sequence content has been enriched by specific sequence amplification.

4. A method as claimed in claim 3 wherein the specific sequence amplification is by polymerase chain reaction amplification (PCR) or ligase chain reaction (LCR).

5. A method as claimed in claim 1, claim 3 or claim 4 wherein the fluorescently detectable agent is a fluorescently detectable oligonucleotide which is capable of hybridizing with the hybridized characteristic sequence of step (ii).

6. A method as claimed in claim 1, claim 3, claim 4 or claim 5 wherein the fluorescently detectable agent is capable of acting as a specific sequence amplification primer, to yield fluorescently detectable amplification product, which is detectable by evanescence when hybridized to the waveguide immobilised oligonucleotide.

7. A method as claimed in claim 6 wherein the specific sequence amplification primer is a polymerase chain reaction primer.

8. A method as claimed in claim 1 or claim 5 comprising:

(i) providing an oligonucleotide, complementary to part of an oligonucleotide sequence characteristic of the RNA or DNA of the material to be detected, immobilised on the surface of an evanescent detector apparatus waveguide;

(ii) providing a fluorescently detectable oligonucleotide complementary to all or part of the remainder of the characteristic sequence;

(iii) exposing the immobilised oligonucleotide from (i) to a solution comprising material to be investigated, or DNA or RNA material derived therefrom, under conditions whereby characteristic oligonucleotide sequence will hybridize with it;

(iv) replacing the solution of material from (iii) with a solution comprising the fluorescently detectable complementary oligonucleotide from (ii) and

(v) measuring the amount of bound fluorescently detectable oligonucleotide using the evanescent wave detector apparatus and relating that to the presence, identity and/or amount of target material in the sample.

9. A method as claimed in claim 1, claim 6 or claim 7 comprising:

(i) providing an oligonucleotide, complementary to part of an oligonucleotide sequence characteristic of the RNA or DNA of the material to be detected, immobilised on the surface of an evanescent wave detector apparatus waveguide;

(ii) providing a fluorescently detectable oligonucleotide identical to all or part of the remainder of the characteristic sequence;

(iii) performing a specific sequence amplification reaction on a sample of the material under investigation using the fluorescently detectable oligonucleotide of step (ii) as one of the primers, to thereby amplify any of the characteristic sequence present while incorporating fluorescently detectable agent therein;

(iv) exposing the waveguide immobilised complementary oligonucleotide of step (i) to the reaction mixture from step (iii) under conditions whereby hybridization of the amplification product with said immobilised oligonucleotide will occur;

(v) measuring any fluorescently detectable oligonucleotide hybridized with the immobilised oligonucleotide using the evanescent wave detector and relating that to the amount of amplified material initially present in the sample.

10. A method as claimed in any one of the aforesaid claims wherein the waveguide comprisies a prism, slab or fibre.

11. A method as claimed in any one of the preceding claims wherein the waveguide is situated within a temperature controllable environment.

12. A method as claimed in claim 11 wherein said environment is a temperature regulated chamber.

13. A method as claimed in wherein the temperature is controllable such that the stringency of the hybridization of the complementary sequences to the target RNA or DNA may be controlled, or such that the

polymerase chain reaction denaturation, hybridization and extension temperature cycles might be carried out.

14. A method as claimed in any one of the preceding claims wherein the fluorescently detectable complementary oligonucleotides are provided by tagging the respective oligonucleotides with a fluorescent label, with a moiety to which a fluorescent species can be bound or with an enzyme or catalyst capable of generating fluorescent species.

15. A method as claimed in claim 14 wherein the complementary oligonucleotide is tagged with fluorescein.

16. A method as claimed in claim 14 wherein the oligonucleotide is tagged with an enzyme or catalyst capable of generating fluorescent species and prior to reading fluorescence with the evanescent wave detector apparatus substrate for this enzyme or catalyst is added.

17. A method as claimed in any one of the preceding claims wherein the material is pretreated with restriction endonucleases to cleave its RNA or DNA.

18. A method as claimed in any one of the preceding claims wherein the complementary or identical oligonucleotides are independently selected from base lengths of 5 to 30 bases long.

19. A method as claimed in claim 18 wherein the oligonucleotides are from 10 to 25 bases long.

20. A method as claimed in claim 19 wherein the oligonucleotides are 15 to 20 bases long.

21. An assay kit for use in the method of claims 1, 2, 3, 4, 14, 15, 16 or 17 comprising:

(A) an oligonucleotide complementary to part of an oligonucleotide sequence, characteristic of the RNA or DNA of the material to be detected, identified and/or quantified, immobilised on the surface of an evanescent wave detector apparatus waveguide and (B) a fluorescently detectable intercalating dye.

22. An assay kit for use in the method of claims 1, 3, 4, 5, 8, 14, 15, 16, or 17 comprising:

(A) an oligonucleotide, complementary to part of an oligonucleotide sequence characteristic of the RNA or DNA of the material to be detected, identified and/or detected, immobilised on the surface of an evanescent wave detector apparatus waveguide, and (B) a fluorescently detectable oligonucleotide complementary to all or part of the remainder of the characteristic sequence.

23. An assay kit for use in the method of claims 1, 3, 4, 5, 6, 7, 9, 14, 15, 16 or 17 comprising:

(A) an oligonucleotide, complementary to part of an oligonucleotide sequence characteristic of the RNA or DNA of the material to be detected, identified and/or detected, immobilised on the surface of an evanescent wave detector apparatus waveguide, and (B) a fluorescently detectable oligonucleotide identical to all or part of the remainder of the characteristic sequence.

24. An assay kit for use in any one of the method claims 1 to 20 comprising oligonucleotides complemetary to all or part of an oligonucleotide sequence characteristic of the RNA or DNA of the material to be detected, identified and/or quantified, together with the reagents necessary for immobilising this to an evanescent wave detector apparatus waveguide.

25. A waveguide suitable for use in an evanescent wave biosensor characterised in that it comprises, immobilised upon its surface, an oligonucleotide which is complementary to all or part of an oligonucleotide sequence which is characteristic of the RNA or DNA sequence of a material which is desired to be detected, identified and/or quantified.

26. An evanescent wave detector biosensor characterised in that it comprises a temperature controllable environment for reception of the evanescent waveguide, suitable for controlling hybridization stringency and/or effecting polymerase chain reaction temperature cycles.

27. An evanescent wave detector bisensor as claimed in claim 26 characterised in that the waveguide has an oligonucleotide, which is complementary to all or part of an oligonucleotide sequence which is characteristic of the RNA or DNA sequence of a material which is desired to be detected, identified and/or quantified, immobilised on its surface.

Fig.1

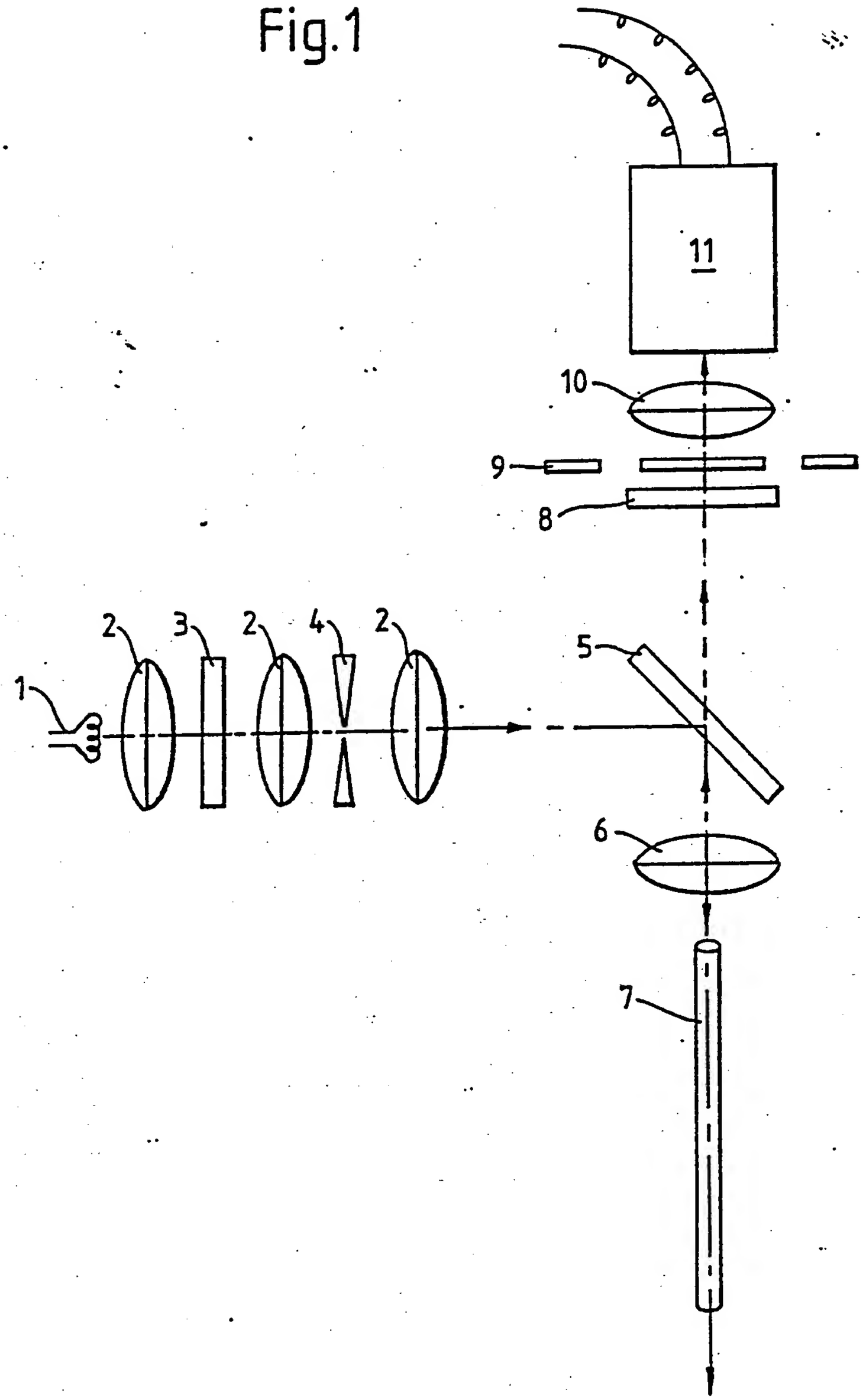


Fig. 2.

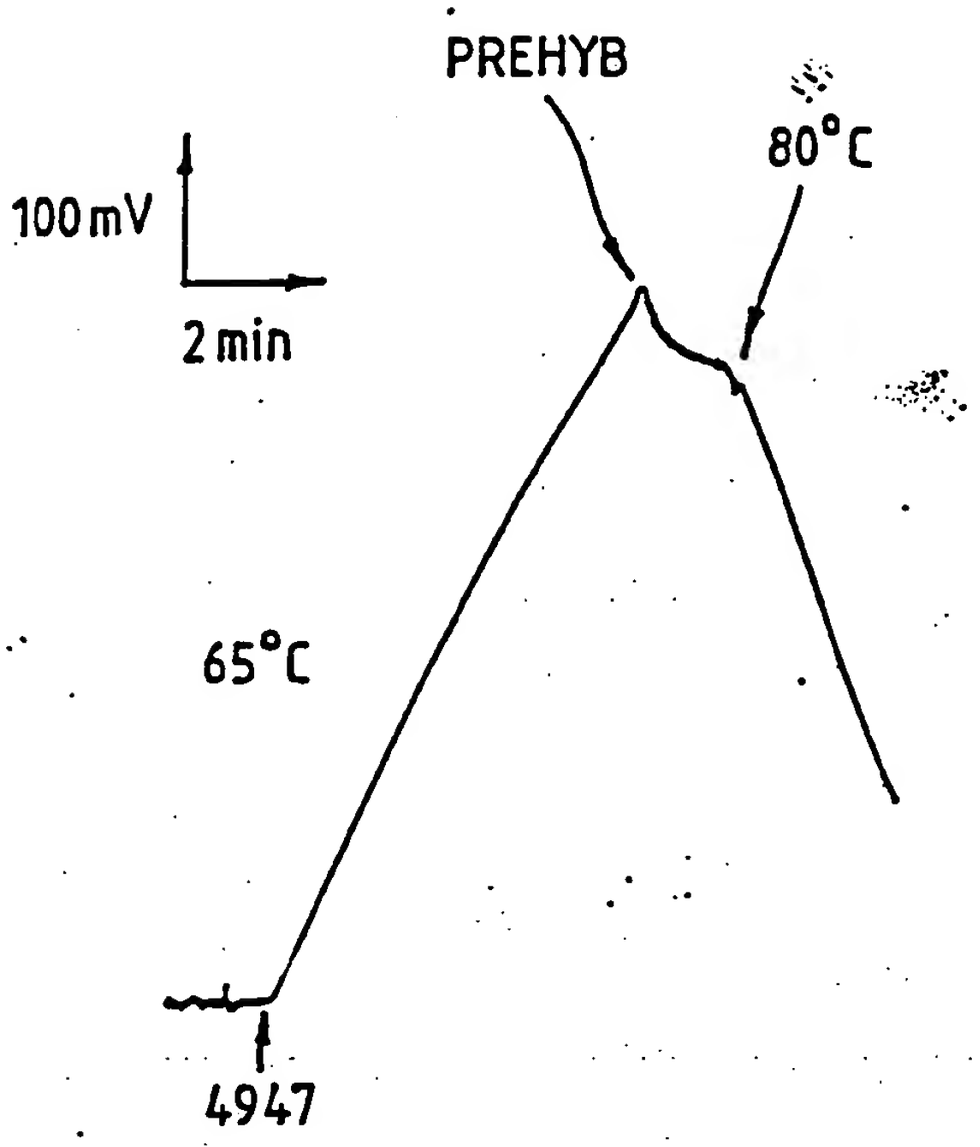

PREHYB
80°C
100 mV
2 min
65°C
4947

Fig. 3.

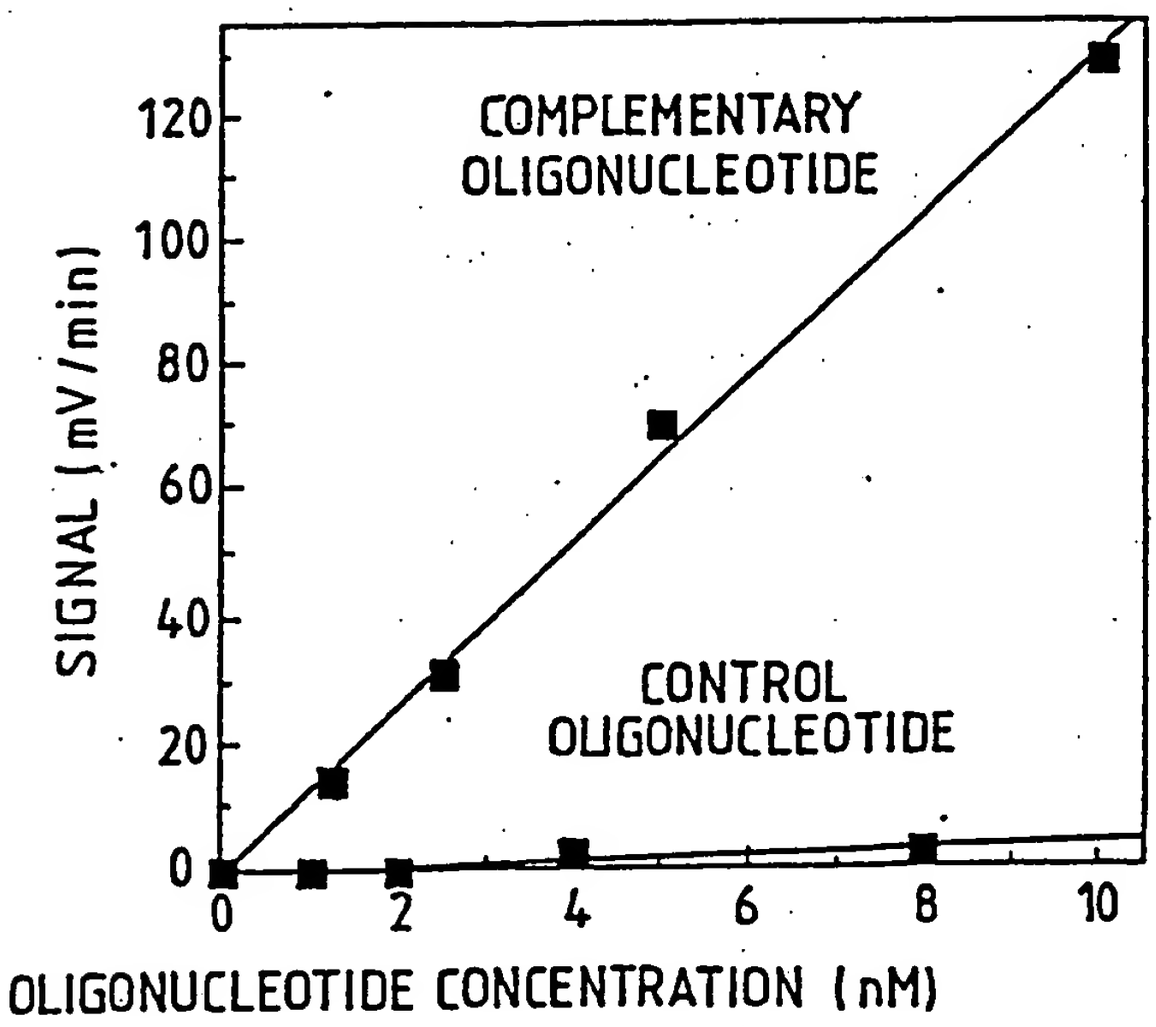

COMPLEMENTARY
OLIGONUCLEOTIDE
CONTROL
OLIGONUCLEOTIDE
SIGNAL (mV/min)
OLIGONUCLEOTIDE CONCENTRATION (nM)
0
20
40
60
80
100
120
0
2
4
6
8
10

Fig. 4.

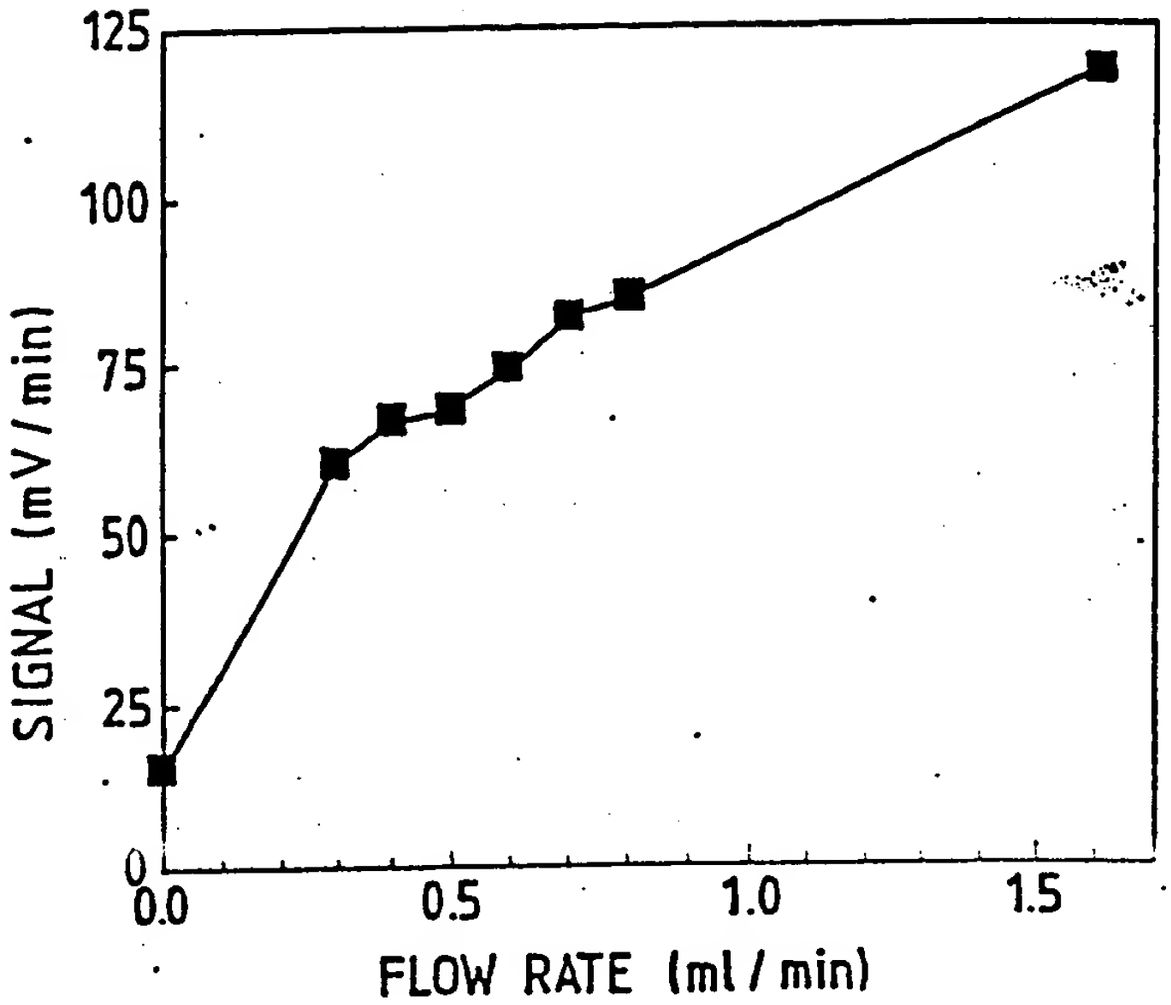

125
100
75
50
25
0
SIGNAL (mV/min)
0.0
0.5
1.0
1.5
FLOW RATE (ml / min)

Fig. 5.

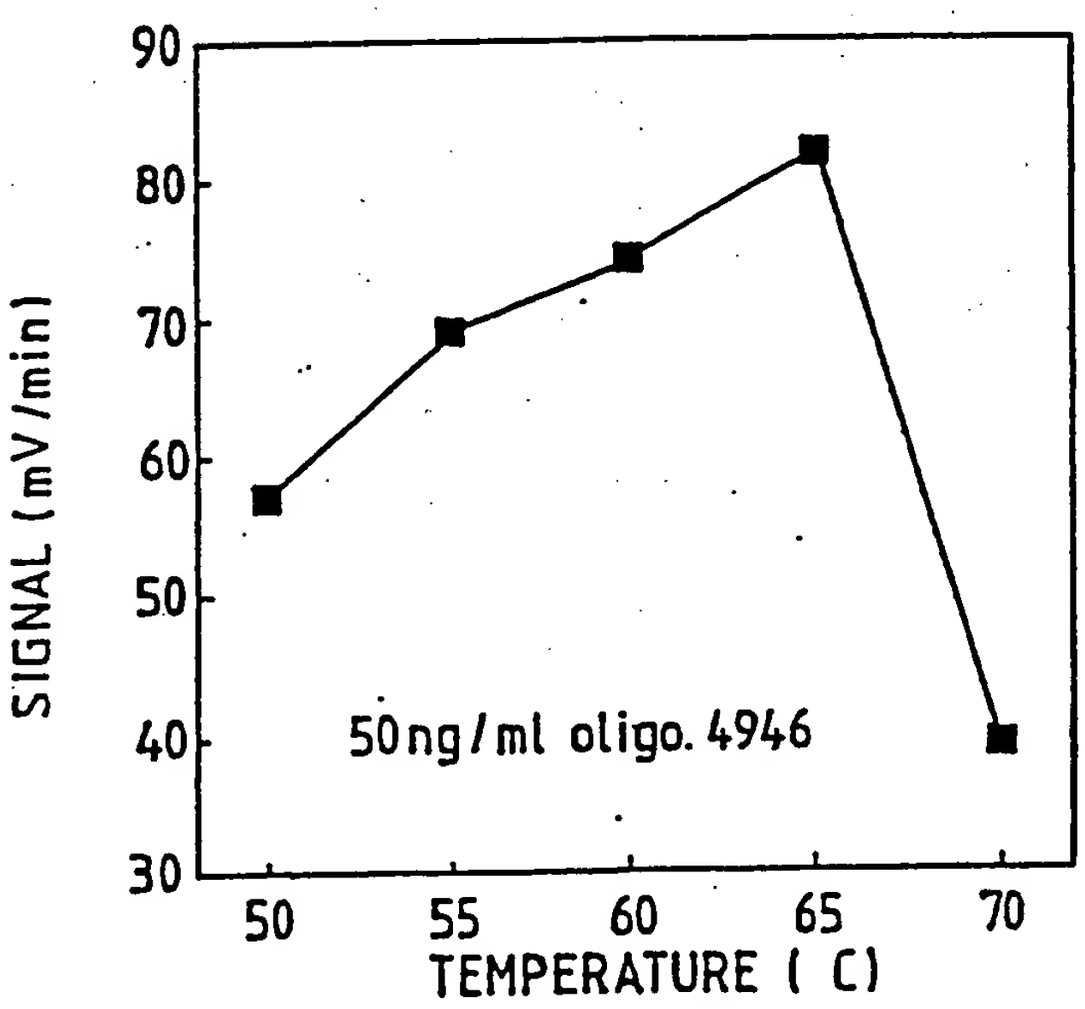

90
80
70
60
50
40
30
SIGNAL (mV/min)
50ng/ml oligo. 4946
50
55
60
65
70
TEMPERATURE (C)

Fig. 6.

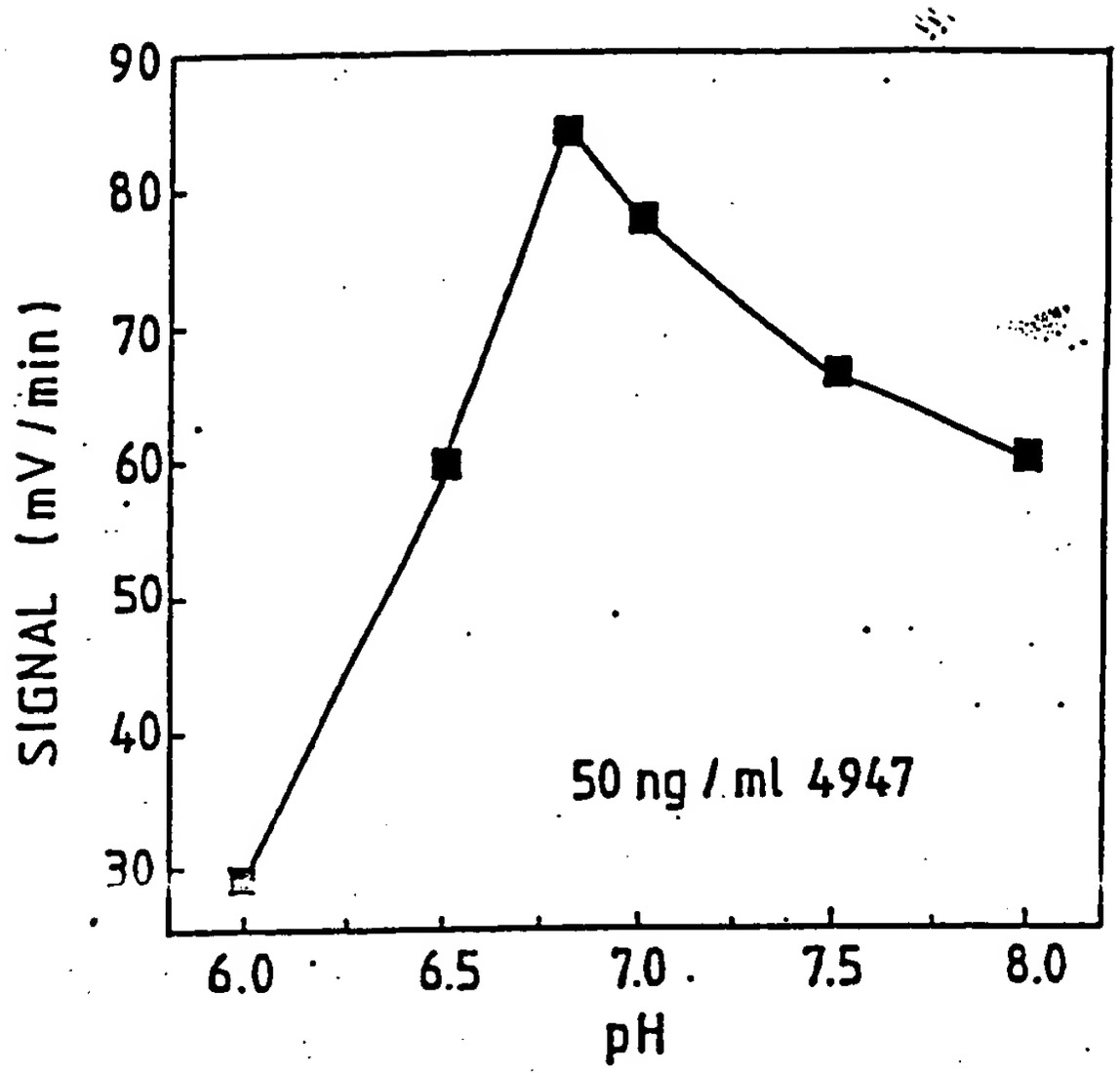
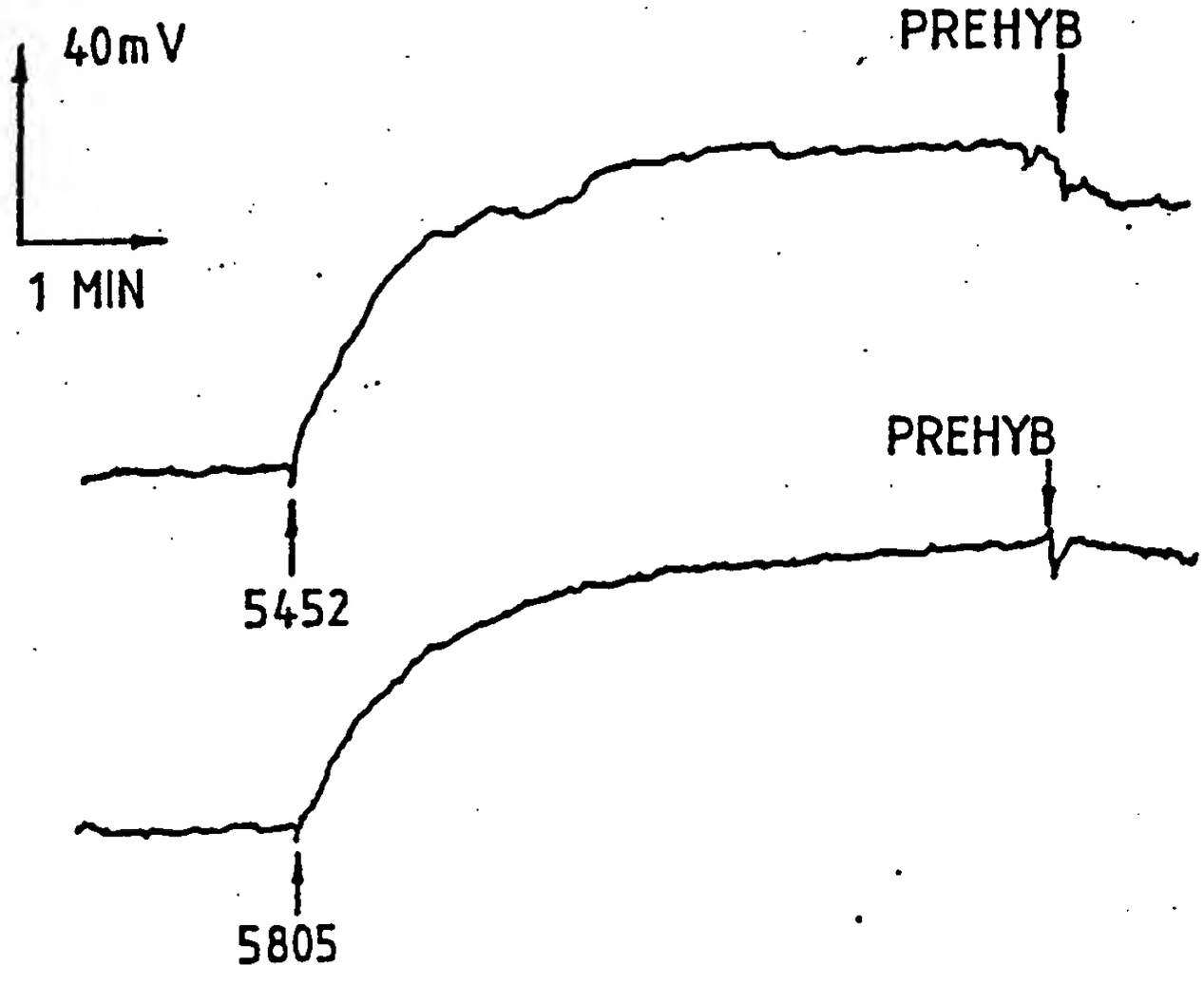

90
80
70
60
50
40
30
SIGNAL (mV/min)
50 ng / ml 4947
6.0
6.5
7.0
7.5
8.0
pH

Fig. 7.


40mV
1 MIN
PREHYB
5452
PREHYB
5805

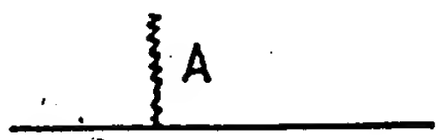


Fig.8a.

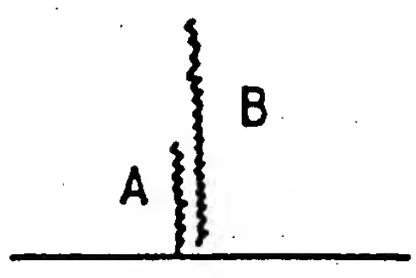


Fig.8b.

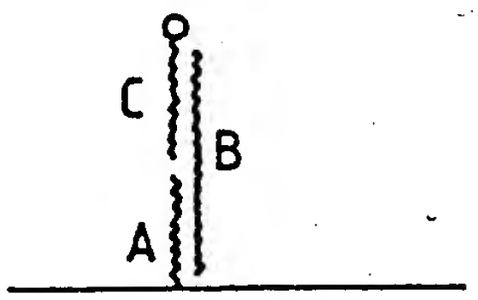


Fig.8c.

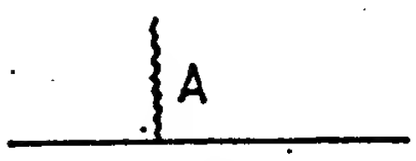


Fig.8d.

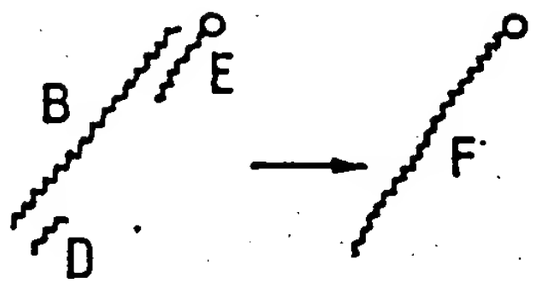


Fig.8e.

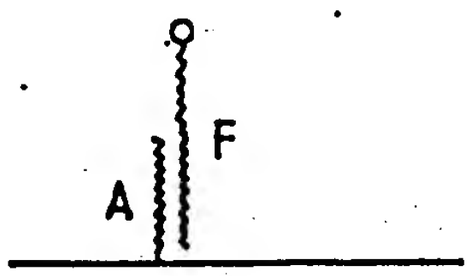


Fig.8f.

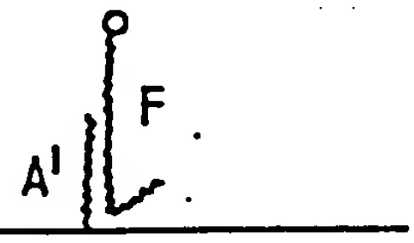


Fig.8h.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/01698

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/68; G01N33/543; G01N21/77

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X Y	EP,A,0 245 206 (BATTELLE MEMORIAL INST.) 11 November 1987 see claims	1,2,10, 21 8,9,26, 27
Y	EP,A,0 070 687 (STANDARD OIL CO.) 26 January 1983 see claims	8
Y	EP,A,0 144 914 (MILES LABORATORIES) 19 June 1985 see claims	8
Y A	EP,A,0 435 150 (ENZO BIOCHEMISTRY) 3 July 1991 see abstract; claims; figures	9,26,27 3,4,6,7, 11-13

-/--

* Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15 DECEMBER 1992	15. 01 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MOLINA GALAN E.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	BE,A,1 000 572 (M. J. BLOCK) 7 February 1989 see claims	1,25
A	WO,A,9 013 666 (AMERSHAM INTERNATIONAL PLC) 15 November 1990 see abstract	1
A	WO,A,9 102 981 (AMERSHAM INTERNATIONAL PLC) 7 March 1991	
P,X	EP,A,0 478 319 (TOSHIBA K.K.) 1 April 1992 see the whole document	1,2,10, 21,25

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9201698 SA 64520

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 15/12/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0245206	11-11-87	AU-A-	7583887	01-12-87
		WO-A-	8706956	19-11-87
		JP-T-	1500221	26-01-89
EP-A-0070687	26-01-83	CA-A-	1180647	08-01-85
		JP-A-	58040099	08-03-83
EP-A-0144914	19-06-85	AU-B-	578436	27-10-88
		AU-A-	3656084	20-06-85
		AU-B-	578641	03-11-88
		AU-A-	3656184	20-06-85
		AU-B-	579335	24-11-88
		AU-A-	3656284	20-06-85
		AU-B-	580745	02-02-89
		AU-A-	3656384	20-06-85
		CA-A-	1250230	21-02-89
		CA-A-	1239580	26-07-88
		CA-A-	1250232	21-02-89
		CA-A-	1238575	28-06-88
		EP-A-	0144913	19-06-85
		EP-A-	0146039	26-06-85
		EP-A,B	0146815	03-07-85
		JP-A-	60151559	09-08-85
		JP-A-	60201256	11-10-85
		JP-A-	60201257	11-10-85
		JP-A-	60179657	13-09-85
		US-A-	4563417	07-01-86
EP-A-0435150	03-07-91	JP-A-	4141099	14-05-92
BE-A-1000572	07-02-89	None		
WO-A-9013666	15-11-90	EP-A-	0471732	26-02-92
		JP-T-	4505251	17-09-92
WO-A-9102981	07-03-91	EP-A-	0489074	10-06-92
EP-A-0478319	01-04-92	None		

EPO FORM P0479